Specialised stomatal humidity responses underpin ecological diversity in C_3 bromeliads

Running title: Stomatal humidity responses in bromeliads

Jamie Males* & Howard Griffiths

Department of Plant Sciences, University of Cambridge

Downing Street, Cambridge, CB2 3EA, United Kingdom

*Author for correspondence

Email: jom23@cam.ac.uk

Telephone: +44 (0)1223 330218

Abstract

The Neotropical Bromeliaceae display an extraordinary level of ecological variety, with species differing widely in habit, photosynthetic pathway, and growth form. Divergences in stomatal structure and function, hitherto understudied in treatments of bromeliad evolutionary physiology, could have been critical to the generation of variety in ecophysiological strategies among the bromeliads. Since humidity is a key factor in bromeliad niches, we focussed on stomatal responses to vapour pressure deficit (VPD). We measured the sensitivity of stomatal conductance and assimilation rate to VPD in eight C_3 bromeliad species of contrasting growth forms and ecophysiological strategies, and parameterised the kinetics of stomatal responses to a step-change in VPD. Notably, three tank-epiphyte species displayed low conductance, high sensitivity and fast kinetics relative to the lithophytes, while three xeromorphic terrestrial species showed high conductance and sensitivity but slow stomatal kinetics. An apparent feedforward response of transpiration to VPD occurred in the tank-epiphytes, while water-use efficiency was differentially impacted by stomatal closure depending on photosynthetic responses. Differences in stomatal responses to VPD between species of different ecophysiological strategies are closely linked to modifications of stomatal morphology, which we argue has been a pivotal component of the evolution of high diversity in this important plant family.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.13024

The bromeliad family displays a diverse array of growth forms and ecophysiological strategies. The extent to which this diversity is underpinned by differences in stomatal behaviour is poorly understood. We quantified stomatal sensitivity to humidity and stomatal kinetics in response to changes in humidity for eight species of contrasting ecologies and stomatal morphologies. Interspecific variation in stomatal responses to humidity clearly aligned with divergences in habit and habitat affinity.

Keywords

Bromeliaceae; stomatal kinetics; stomatal sensitivity; VPD; humidity; stomata; transpiration

Introduction

The Bromeliaceae is a diverse monocot family of a predominantly Neotropical distribution and some 3,500 spp. (Butcher & Gouda, 2017). The bromeliads encompass an impressive range of growth forms and ecologies (Benzing, 2000), and ongoing research efforts are gradually revealing how this variety has arisen from both divergent and convergent evolution of anatomical, morphological and physiological traits (Givnish et al., 2014; Palma-Silva et al., 2016; Males & Griffiths, 2017a). Special emphasis has been placed on a suite of putative key innovations, some of which appear to have driven enhanced diversification rates in certain lineages and perhaps facilitated adaptive radiations (Schulte et al., 2009; Givnish et al., 2011, 2014; Silvestro et al., 2014). These innovations include absorptive foliar trichomes, epiphytism, Crassulacean acid metabolism (CAM), and water- and nutrient- impounding 'tanks' formed by overlapping leaf-bases. The functional variety in the Bromeliaceae is underpinned by various combinations of these innovations, which are associated with distinctive ecophysiological strategies (Males & Griffiths, 2017a). The bromeliads therefore offer an excellent context for investigating how structure-function relationships have affected the evolution of ecological diversity in an important angiosperm radiation (Males, 2016).

Besides the well-studied key innovations that occur in the Bromeliaceae, a whole host of other leaf traits likely to be of profound relevance to ecological specialisation have received comparatively little attention (Males, 2016). Among these is the sensitivity of the stomatal apparatus to microenvironmental factors, which affects gas exchange by modulating stomatal conductance to water vapour (g_{sw}) and CO_2 (g_{sc}). In some cases, stomatal sensitivity can generate 'apparent feedforward' responses of transpiration rate (E) to VPD, in which the decline in g_{sw} in response to VPD eventually causes E to decline also, despite the increase in driving force. Some explanations proposed for feedforward phenomena involve direct, perhaps hydropassive, sensing of humidity around the stomatal pore (Lange et al., 1971; Farquhar, 1978; Peak & Mott, 2011). By contrast,

indirect feedback mechanisms, which are dependent on a change in leaf water status to elicit a reduction in stomatal aperture, have also been theoretically and empirically implicated in the generation of feedforward effects (Bunce, 1997; Buckley, 2005; Bauer et al., 2013; McAdam & Brodribb, 2015; Martins et al., 2016). The relative importance of direct and indirect effects remains a controversial and active area of research. Regardless of the mechanism, variation in stomatal sensitivity and the potential for feedforward effects is known to differ widely within other angiosperm groups (e.g. Morison & Gifford, 1983; Oren et al., 1999), and could be an important determinant of habitat preferences in the bromeliads. In water-limited ecosystems or microhabitats, or in situations characterised by frequent fluctuations in light and humidity levels, strong, rapid stomatal responses might facilitate close tracking of extreme and/or dynamic environmental conditions. This scenario might apply specifically to tank-epiphyte species, which have limited internal water storage and must therefore display conservative stomatal behaviour, and especially those species which inhabit more water-limited or seasonal environments where the tank may often be empty (Males & Griffiths, 2017a). Conversely, close environmental tracking may be less important for soil-rooted terrestrial mesophytes which have access to a more constant supply of moisture. Meanwhile, other terrestrial species are lithophytic and may be subject to acute or prolonged seasonal water limitation, they are nevertheless known to display relatively high leaf hydraulic conductance and transpiration rates, suggestive of adaptation to episodically high levels of water use (Males & Griffiths, 2017a). While no systematic survey has yet been performed to investigate the relevance of stomatal sensitivity to ecological differentiation in the bromeliads, there have been a few sporadic reports of strong sensitivity to humidity in the literature (Lange and Medina, 1979; Adams and Martin, 1986; Lüttge et al., 1986a). These studies suggested that the stomata of at least some bromeliad species displayed an apparent feedforward response.

Alongside stomatal sensitivity, stomatal kinetics are increasingly recognised as another important determinant of overall intrinsic water-use efficiency (WUE; A/g_{sw}). There have been several important recent methodological and theoretical advances in stomatal signalling and kinetics (e.g. Bauer et al., 2013; McAdam & Brodribb, 2015; McAdam et al., 2016; McAusland et al., 2016; Vialet-Chabrand et al., 2016). When a plant at steady-state g_{sw} is exposed to a step-wise increase in leaf-air vapour pressure deficit (VPD), g_{sw} typically displays a transient increase known as a 'wrong-way response' (WWR). The WWR remains an enigmatic phenomenon, with ongoing research suggesting a role for differential localised responses of plasma membrane aquaporin (PIP) activity (Nonami et al., 1991), but is generally attributed to the mechanical advantage of the epidermis (Mott and Franks, 2001; Buckley et al., 2003, 2011; Buckley, 2005; McAdam & Brodribb, 2015; Wolf et al., 2016). The WWR's physiological significance is quantifiable in terms of the volume of excess water

loss that it engenders (Lawson and Blatt, 2014). This is followed by a gradual decline in g_{sw} until a new, lower steady-state is attained. This 'right-way response' (RWR) minimises water loss under increased evaporative demand, thereby reducing the chances of a physiologically challenging drop in leaf water potential (Saliendra et al., 1995). Preservation of high leaf water potential is especially important in the bromeliads, which display limited investment in osmotically-mediated drought tolerance (Males & Griffiths, 2017a). However, because stomatal aperture affects not only water loss but also net CO_2 assimilation (A), the RWR to increased VPD restricts the supply of CO_2 for photosynthetic assimilation. Depending on the relative importance of stomatal, mesophyll and biochemical limitations on photosynthesis (*sensu* Grassi & Magnani, 2005), A may therefore decrease in concert with reductions of stomatal aperture caused by elevated VPD. The degree of stomatal sensitivity to VPD can thus have important consequences for plant carbon and water economies, with different possible outcomes for WUE.

We consider this a timely moment at which to contextualise interspecific variation in stomatal sensitivity by examining links with ecological syndromes. While the ecophysiological diversity of the bromeliads make them an excellent study system, the different stomatal complex morphologies found in the family also provides opportunities to seek insights into structural contributions to variation in stomatal sensitivity and kinetics (Tomlinson, 1969; Males, 2016). In particular, the presence of specialised subsidiary (and hypodermal) cells in certain bromeliad lineages could be of functional significance given the importance of these cells in defining stomatal movements in other plant groups (Johnsson et al., 1976; Franks & Farquhar, 2007; Chen et al., 2017).

Based on the foregoing considerations, we set out to test five key hypotheses:

1. Stomatal sensitivity to VPD should be weaker in xeromorphic terrestrial species where high hydraulic capacitance can buffer transpiration.
2. Stomatal sensitivity to VPD should be stronger in epiphytic than in terrestrial species as a component of adaptation to water limitation in epiphytic niches.
3. Rapid stomatal kinetics should co-occur with strong stomatal sensitivity, since both should be selected for in water-limited environments.
4. Stomatal sensitivity to VPD should be stronger in species with high maximal stomatal conductance, as observed across other plant groups (e.g. Oren et al., 1999).
5. Differences in stomatal sensitivity to VPD and stomatal kinetics should be associated with divergences in stomatal complex morphology, given the role of subsidiary cells in other species (Franks & Farquhar, 2007).

To seek support for our hypotheses, we quantified stomatal sensitivity to VPD and the kinetics of stomatal responses to VPD in eight representative C_3 species of contrasting ecophysiological strategies with different stomatal complex morphologies and levels of tissue succulence (see Fig. 1 for images of each species). Two lithophytic species from the genus *Pitcairnia* L'Her. (Pitcairnioideae subfamily) were chosen, which display the simple liliaceous rosette growth form that is plesiomorphic in the Bromeliaceae (Benzing, 2000). Three species from the genera *Ochagavia* Phil. (Bromelioideae) and *Puya* Molina (Puyoideae) were used. These species are more canonical xeromorphic terrestrials, with thickened, strongly spinose leaves. Finally, three species from the genera *Guzmania* Ruiz & Pav. (Tillandsioideae), *Lutheria* Barfuss & W.Till (Tillandsioideae), and *Nidularium* Lem. (Bromelioideae) were used. These species, while differing in habitat affinity, are obligate or facultative tank-epiphytes, in which the combination of a water-impounding tank and efficient absorptive trichomes facilitates survival in epiphytic habitats. By identifying clear divergences in stomatal responses to VPD between species with contrasting stomatal morphology, our results cast light on an overlooked aspect of structural-functional environmental specialisation in a major plant radiation.

[FIGURE 1]

Materials and methods

Plant material

Eight C_3 bromeliad species expemplifying different ecophysiological strategies were selected for use in this investigation. Species selection was strongly phylogenetically structured due to the serial evolutionary progression in the Bromeliaceae from terrestrial to epiphytic habits. Two lithophytic *Pitcairnia* species (Pitcairnioideae), with small, simple anomocytic stomata were chosen: *P. integrifolia* Ker Gawl., a soft-leaved species with a disjunct distribution in moist forests of Trinidad, northern Venezuela and southeastern Brazil, and *P. xanthocalyx* Mart., which is similar in morphology and habit, but native to tropical dry forest in Mexico. Although these species occur in water-limited microhabitats, especially in the dry season, they clearly function as relatively highly productive water-spenders when water is available (Males & Griffiths, 2017a). Three xeromorphic terrestrial species were included: *Ochagavia elegans* Phil. (Bromelioideae), a semi-saxicolous species native to coastal scrub on Juan Fernandez Island, Chile; *Puya berteroniana* Mez (Puyoideae), a large C_3-CAM xerophyte from Mediterranean-biome scrub in central Chile; and *P. mirabilis* (Mez) L.B.Sm. (Puyoideae), a morphologically-reduced strictly C_3 species native to more arid scrub in Bolivia and

north-western Argentina. In *O. elegans* and both *Puya* species, the large guard cells are subtended by two lateral subsidiary cells that appear to restrict effective pore aperture. Three tank-epiphyte species were chosen: *Guzmania monostachia* (L.) Rusby ex Mez (Tillandsioideae), a C_3-CAM intermediate species with a wide distribution in evergreen and seasonally-deciduous forest across the Caribbean, Central America and north-eastern South America; and *Lutheria glutinosa* (Lindl.) Barfuss & W.Till (Tillandsioideae), a facultative epiphyte with a poorly-developed tank that occurs in montane forest in Trinidad and Venezuela; and *Nidularium innocentii* Lem. (Bromelioideae), a C_3, thin-leaved, facultative epiphyte from a predominantly CAM-performing genus native to the Atlantic Forest of Brazil. In all three tank-epiphyte species (*G. monostachia*, *L. glutinosa*, and *N. innocentii*), large hypodermal cells protrude into the substomatal cavity (Tomlinson, 1969). These cells can effectively occlude the pore, potentially providing a considerable resistance to gas exchange (Tomlinson, 1969).

Table 1 summarises the species used in this investigation.

[TABLE 1]

All plant material was sourced from the living collection of Cambridge University Botanic Garden (CUBG), UK. With the exception of the xeromorphic terrestrials, all species were grown in a tropical glasshouse with daytime temperature of 24-30°C, night-time temperature of 18-24°C, and relative humidity of 85-100%. *O. elegans* was grown in a subtropical glasshouse, with daytime temperature of 16-28°C, night-time temperature of 12-18°C, and RH of 50-80%. Through a combination of natural and artificial illumination, all plants in the glasshouses received a minimal daytime photosynthetic photon flux density (PPFD) of at least 300 µmol m^{-2} s^{-1}, which is known to be above saturating for all of these species (Males & Griffiths, 2017a). The two *Puya* spp. were grown outdoors in Cambridge's warm temperate climate. Historical climate data for CUBG are available online at http://www.botanic.cam.ac.uk/Botanic/Page.aspx?p=27&ix=2830. For all species, only mature plants were used for anatomical and physiological characterisation; possible ontogenetic changes in stomatal physiology (e.g. Pantin et al., 2013a,b) were beyond the scope of the current investigation, but are considered in the Discussion.

Stomatal pore length and density

Epidermal impressions of the abaxial surface (hypostomatous leaves are almost universal in the Bromeliaceae; Tomlinson, 1969) of the central portions of 30 fully-expanded leaves of each species were taken using nail varnish, sampling from at least three individuals per species. For the purposes of comparability, the same central portion of the leaf was sampled as was used for gas exchange measurements, although in some species there may be higher density in the apical portion (e.g. Freschi et al., 2010). Stomatal pore length and stomatal density were measured by light microscopy, the latter across regions of at least 10 mm x 10 mm.

Stomatal aperture

To verify the coupling of guard cell movements with changes in g_{sw} measured by gas exchange, a total of 20 fully-expanded leaves of each species were detached from at least three individuals and incubated under a universally-saturating photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} at intervals of leaf-air vapour pressure deficit (VPD) ranging from 0.02 kPa to 2.2 kPa. After at least 45 minutes of acclimation at each VPD level, epidermal impressions of the abaxial surface of the leaves were taken and observed under a light microscope. Stomatal aperture (α) was quantified as the maximum diameter of the pore between pairs of guard cells. The instantaneous sensitivity of α to VPD (S_α) was calculated as -dα/dlnVPD, and normalised by the extrapolated maximal stomatal aperture (α_{max}).

Gas exchange measurements

Fully-expanded leaves in the second rosette layer were selected for measurements of gas exchange using a LI-6400-XT infra-red gas analyser fitted with the standard 2x3 cm LED-supplied leaf chamber (LI-COR, Lincoln, Nebraska, USA). An external CO_2 concentration of 400 ppm and a PPFD of 350 µmol m^{-2} s^{-1} was supplied in all gas exchange measurements, and constant leaf temperature of 25°C ± 0.01°C was maintained throughout. The sample sizes for VPD response curves and VPD step-change analysis are given below. During preliminary measurements, leaf water potential readings were taken using a 600D pressure chamber (PMS Instruments, Albany, OR, USA) at the beginning and end of the VPD response curves and VPD step-change measurements. The final leaf water potential never fell below -0.30 MPa, suggesting that the VPD response was unlikely to have been significantly confounded by a change in leaf water status (Males & Griffiths, 2017a).

VPD response curves

Five replicate VPD response curves were performed for each species using leaves from at least three individuals and the LI-6400XT to measure gas exchange. VPD was varied by altering absolute water content of the air while holding leaf temperature constant (Buckley, 2005). Each leaf was allowed to acclimate at a VPD of 0.2 kPa until g_{sw} reached steady state (approximately one hour). Gas exchange variables (A, E, g_{sw}, WUE) were logged at 30-second intervals for five minutes. The air inlet stream was then dehumidified to attain a VPD of 0.5 kPa, and the leaf was allowed to acclimate until a new steady-state g_{sw} was reached and gas exchange variables were logged again. This process was repeated at VPD levels of 0.8 kPa, 1.1 kPa, 1.4 kPa, 1.7 kPa, and 2.2 kPa. The maximum theoretical stomatal conductance (g_{swmax}) was extrapolated from the measured data. The instantaneous sensitivity of g_{sw} to VPD was calculated as $-dg_{sw}/d\ln VPD$ (Oren et al., 1999) and normalised by g_{swmax}. The slope of this relationship is constant and therefore useful for interspecific comparison.

VPD step-change analysis

Eight replicate VPD step-change responses were recorded for each species, using leaves from at least three individuals. Each leaf was allowed to acclimate at a VPD of 0.5 kPa until g_{sw} reached steady state (approximately one hour). Gas exchange variables were continuously recorded at 120 s intervals from this point onwards. After 10 minutes of logging at steady-state g_{sw}, the air-inlet stream was immediately switched to a dehumidified stream to increase VPD to 0.8 kPa. The response of g_{sw}, E, and A to this step-change in VPD was monitored and logging continued until the WWR and subsequent RWR were completed with the arrival at a new steady state.

Statistics

All statistical analyses were performed in R (R Core Development Team, 2008). For VPD response curves, non-linear curve-fitting was performed using exponential or polynomial functions as detailed in figure legends.

Results

[FIGURE 2]

Stomatal anatomy, aperture and sensitivity

Examples of epidermal impressions are displayed in Fig. 2, which highlights the differences in stomatal size, stomatal density, and stomatal complex morphology between species. Stomatal size and density varied considerably within our species set (Table 2). The smallest stomata were observed in the two lithophytic *Pitcairnia* species (< 12 µm pore length), which also showed by far the highest stomatal density (> 400 mm^{-2}). Larger (> 28 µm) stomata at lower density (< 100 mm^{-2}) were observed in the remaining species. The two species from the Bromelioideae subfamily, *O. elegans* (xeromorphic terrestrial) and *N. innocentii* (tank-epiphyte) showed the lowest stomatal densities (< 20 mm^{-2}).

[TABLE 2]

Maximal stomatal aperture (α_{max}) was highest in the xeromorphic terrestrials and lowest in the tank-epiphytes. There was no significant correlation between pore length and α_{max} (n = 8, r^2 = 0.29, p = 0.17), suggesting that differences in lateral extensibility of guard cells are more important in driving variation in effective pore area. Normalised species-specific responses of α to VPD are displayed in Fig. 3. The decline in α with increasing VPD was distinctly stronger in the three tank-epiphytes (n = 8, F = 78.84, p < 0.001), as reflected in the high values of the instantaneous sensitivity of stomatal aperture to VPD (S_α) calculated for these species (Table 2). Across all species, there was a statistically significant negative linear correlation between α_{max} and S_α; n = 8, r^2 = 0.52, p = 0.04).

[FIGURE 3]

Stomatal conductance

The highest maximal stomatal conductance to water vapour (g_{swmax}) occurred in the xeromorphic terrestrial species (> 0.220 mol m^{-2} s^{-1}), whereas the lowest values were recorded in the tank-epiphytes (< 0.140 mol m^{-2} s^{-1}; Table 3). Across all eight species, there was a strong positive correlation between α_{max} and g_{swmax} (n = 8, r^2 = 0.87, p < 0.01). In accordance with the stomatal aperture measurements, all species examined showed the expected decline of steady-state g_{sw} in response to increasing VPD (Fig. 4A). The strengths of the responses of each species, expressed as the instantaneous sensitivity of stomatal conductance to VPD (S_S), are displayed in Table 3. The

highest values of S_S occurred in the tank-epiphytes (> 0.180), indicating that stomata of these species are characterised by strong responsiveness to VPD. Meanwhile S_S did not differ between lithophytes and xeromorphic terrestrials (n = 5, F = 0.01, p = 0.913). S_S was positively correlated with S_α (n = 8, r^2 = 0.74, p < 0.01), although this relationship was driven by the distinctiveness of the tank-epiphyte species, which combined high S_S with high S_α. Interestingly, g_{swmax} and S_S were negatively correlated (n = 8, r^2 = 0.68, p = 0.01), suggesting that bromeliads with stomata capable of attaining high maximal conductance tend to be less sensitive to VPD.

[TABLE 3]

[FIGURE 4]

Transpiration rates

In the tank-epiphyte species, the reduction in g_{sw} with increasing VPD was sufficient to restrict the increase in transpiration rate (E) that would otherwise occur (Fig. 4B). In *N. innocentii* and *L. glutinosa*, E actually decreased between VPD = 0.60 kPa and VPD = 2.20 kPa, potentially representing a feedforward effect of VPD on E. Meanwhile E remained relatively constant in *G. monostachia* across the same range of VPD, indicative of an isohydric strategy. Conversely, there was a linear increase in E across the measured VPD range among all terrestrial species.

Assimilation rates

Alongside the reduction in g_{sw}, increasing VPD elicited a fall in net assimilation rate (A) in all species, presumably due to the consequent restriction of CO_2 availability for photosynthesis (Fig. 4C). Values of maximal assimilation rate (A_{max}) and the instantaneous sensitivity of assimilation to VPD (S_A) are displayed in Table 3. There was no significant correlation between A_{max} and S_A (n = 8, r^2 = 0.22, p = 0.25), nor was A_{max} correlated with g_{swmax} (n = 8, r^2 = 0.07, p = 0.52). Although not statistically significant (n = 8, F = 2.25, p = 0.184), S_A was higher in the tank-epiphytes (> 0.150), where the restriction of stomatal conductance was strongest. However, across all eight species S_A was not correlated with S_S (n = 8, r^2 = 0.23, p = 0.23), perhaps due to interspecific differences in the strength of stomatal limitations on photosynthesis relative to mesophyll and biochemical limitations (*sensu* Grassi & Magnani, 2005).

Water-use efficiency

Interspecific differences in the response of water-use efficiency (WUE) to VPD (Fig. 4D) were driven largely by differences in the relationship between *A* and VPD. Thus both *N. innocentii* and *O. elegans*, which displayed very strong restriction of *A* in response to VPD, also showed reductions in WUE as VPD increased, whereas all other species showed increases in WUE up to VPD = 1.25 kPa. At levels of VPD higher than 1.25 kPa, WUE began to decline for both lithophytic *Pitcairnia* species and for the tank-epiphyte *L. glutinosa*. The same occurred for the tank-epiphyte *G. monostachia* and the xeromorphic terrestrial *Puya mirabilis* above 1.60 kPa. For the xeromorphic terrestrial *Puya berteroniana*, WUE reached a plateau of 2.7x its initial value at 1.25 kPa, and remained at this level.

Step-change analysis

As expected, a step-increase in VPD from 1.3 kPa to 1.8 kPa elicited a transient wrong-way response (WWR) followed by a decline in g_{sw} to a new, lower steady state in all species (Fig. 5). There was an equivalent decline in *A* in all cases, such that intrinsic WUE was lower in the new steady state for all species except *Puya mirabilis*. This contrasts with the steady-state data presented above, perhaps because adjustments in photosynthetic activity had not been completed across the time period for which non-steady-state gas exchange was recorded. The kinetics of the stomatal response to step-changes varied between species. Interspecific differences in response profiles could be expressed in terms of a set of key parameters, displayed in Table 4. The temporal duration of the WWR, t_{WWR}, was quantified, and found to be lowest in the three tank-epiphyte species (< 1000 s). The highest values of t_{WWR} were recorded in the xeromorphic terrestrial species (> 1900 s). The area under the g_{sw} curve for the duration of the WWR (AUC_{WWR}) was used as an indicator of the amount of additional water lost during initial wrong-way response above the amount of water that would be transpired if the initial steady-state g_{sw} did not change. AUC_{WWR} was highest in the xeromorphic terrestrials (61-219 mol m^{-2}) and lowest in the tank-epiphytes (3-28 mol m^{-2}), indicating that tank-epiphytes lost the lowest absolute quantity of water during the WWR. The close positive correlation between t_{WWR} and AUC_{WWR} across species (n = 8, r^2 = 0.80, p < 0.01) highlights the importance of stomatal kinetics in determining the efficiency of responses to environmental cues. There was a strong negative correlation between t_{WWR} and S_S (n = 8, r^2 = 0.59, p = 0.03), suggesting that species with more constrained VPD-induced WWRs also show the highest sensitivity to VPD in steady-state g_{sw}. Parameters related to the RWR were also quantified for each species. The duration of the right-way response (RWR), t_{RWR}, varied between 1010 s in *P. xanthocalyx* and 2560 s in *P. mirabilis.* Unlike with t_{WWR}, t_{RWR} was not correlated with S_S (n = 8, r^2 = 0.11, p = 0.43). Whereas the lithophytes showed

both low t_{RWR} and low S_S, the tank-epiphytes combined high S_S with low t_{RWR}, while the xeromorphic terrestrials showed high t_{RWR} but low S_S.

[FIGURE 5]

[TABLE 4]

As occurred in the steady-state measurements, the step-change in VPD caused a reduction in *A* in all species, highlighting the critical role of stomata in controlling the dynamics of carbon assimilation in bromeliad leaves. The physiological significance of the WWR effect is reflected in the strong transient decrease in intrinsic WUE observed in all species, where excessive water loss occurs alongside reducing carbon assimilation.

Discussion

The Bromeliaceae offers an ideal system in which to study the role of structure-function relationships in mediating ecological differentiation (Males, 2016). Since a range of complex water-use strategies and contrasting microenvironments are associated with ecological variety in this family, we hypothesised that divergences in stomatal responses to VPD could be a critical physiological contributor to ecological differentiation among the bromeliads. Our characterisation of the steady-state and non-steady-state stomatal responses to VPD of eight contrasting species demonstrates the potential for such divergences to modulate water-use efficiencies and bioclimatic relations. It also provides empirical evidence consistent with the long-conjectured linkage between stomatal morphology and stomatal behaviour across different bromeliad lineages (Tomlinson, 1969).

Comparison of the stomatal properties of the bromeliads studied here with those of other plant groups (e.g. Oren et al., 1999) suggests that maximal stomatal conductance is generally relatively low and sensitivity of steady-state g_{sw} to VPD high in the Bromeliaceae. Data that could help contextualise the kinetics of bromeliad responses to changing VPD are more limited. Martins et al. (2016) showed more rapid stomatal closure in some ferns and conifers in response to a larger step-change in VPD, which they attributed to hydropassive responses controlled by leaf hydraulic traits. The durations of the responses to VPD in the bromeliads were broadly comparable with those measured for stomatal opening in response to light in a range of herbaceous and woody species by McAusland et al. (2016).

Ecological significance of stomatal humidity responses in the Bromeliaceae

Variation in stomatal functional traits among the species used in this investigation can be readily interpreted in the context of the differences in water-use strategies among bromeliad species. Many terrestrial bromeliads have access to the soil water reservoir and are therefore often not subject to the same degree of water-limitation as is common for epiphytic species (Males, 2016). Others, particularly those displaying the lithophytic habit, may experience frequent and severe water limitation. Thus despite the much higher stomatal densities measured in the lithophytic *Pitcairnia* spp. used in this investigation, these species did not display significantly higher maximal stomatal conductance than some tank-epiphytes, and even C_3 terrestrial bromeliads show relatively low gas exchange capacity and hydraulic conductance compared with other plant groups (Males & Griffiths, 2017a). However, steady-state stomatal conductance is less sensitive to VPD in the lithophytes than in tank-epiphytes. Lithophytes always display a certain amount of foliar water-storage tissue, and this internal reservoir may help buffer against changes in transpiration rate induced by variation in VPD. Stomatal sensitivity is nevertheless sufficient for modest increases in VPD to engender a rise in WUE. In terms of stomatal kinetics, the lithophytes studied here appeared to be less conservative than the tank-epiphyte species, losing more water during WWRs, which suggests that these lithophytes may use different physiological strategies to deal with episodic water limitation. However, they did display rapid RWRs in response to step-changes in VPD, perhaps in connection with small stomatal size (see below).

The xeromorphic terrestrial species studied here all show high hydraulic capacitance (Males & Griffiths, 2017a). Consistent with observations in other plant groups (e.g. Martins et al., 2016), this high capacitance in the xeromorphic terrestrial bromeliad species in our study was associated with slow stomatal kinetics. This supports our hypothesis that among terrestrial bromeliads, high capacitance facilitates continued transpiration at higher rates when VPD increases. However, relatively high capacitance, based on thick layers of differentiated water-storage tissue, also occurs in lithophytic species (Males & Griffiths, 2017a). The relationship between tissue-level succulence and stomatal behaviour is therefore not clear-cut, and will require more nuanced investigation in a phylogenetically-explicit framework. Furthermore, the extent to which water-storage tissues can be dynamically coupled to the transpiration stream in succulent plants remains unclear, and more empirical work will be needed to address this important physiological question (Males, 2017). Despite maintaining comparatively high g_{sw} as VPD increased, the xeromorphic terrestrial species used here nevertheless did display a substantial reduction in g_{sw} relative to g_{swmax} across the modest VPD levels to which they were subjected in this investigation. In nature, plants of these species probably frequently experience somewhat higher VPD for much of the day. For example, Giliberto &

Estay (1978) reported summertime VPD maxima of approximately 3.5 kPa on exposed south-facing slopes in central Chilean matorral similar to the habitat of *Puya berteroniana*. These higher levels of VPD could restrict g_{sw} even further, potentially leading to an apparent feedforward effect. An evolutionary consequence may have been selection for high thermal tolerance in these xerophytes, since frequent daytime stomatal closure may strongly diminish the potential for evaporative cooling (Chaves et al., 2016), while one possible physiological consequence would be a midday depression of net CO_2 fixation, as has been observed in other bromeliads (e.g. Griffiths et al., 1986; Lüttge et al, 1986b). The latter effect may be stronger in xeromorphic terrestrials such as *O. elegans*, which experiences strong stomatal limitation of photosynthesis as VPD increases. Net CO_2 uptake could even be primarily limited to fog events, which are known as important ecological occurrences in the littoral habitats on Juan Fernández Island to which *O. elegans* is native (Valdebenito et al., 1992). Zizka et al. (2009) have also remarked on the restriction of some xerophytic *Puya* species to areas of coastal Chile that are frequently subject to fog events. It is nevertheless somewhat surprising that *O. elegans* in particular should show such a strong negative relationship between VPD and WUE given that it is not native to habitats of very high daytime humidity. It is interesting to note that this species shows a borderline C_3-CAM carbon isotope ratio, and the congeneric *O. carnea* (Beer) L.B.Sm. & Looser displays significant day-night rhythmicity in mesophyll cell-sap osmolality when drought-stressed (Crayn et al., 2015; Males & Smith, unpublished). It is also possible that under natural water-availability regimes, long-term acclimation to low soil water potential could lead to altered stomatal responses relative to what was observed in well-watered experimental plants.

The tank-epiphytes of the Bromelioideae and Tillandsioideae subfamily represent a remarkable case of convergent evolution in habit and morphology, and a keystone component of Neotropical forest ecosystems (Benzing, 2000). Alongside other exposure-related stressors, water-limitation is characteristic of epiphytic habitats, and this has selected for the specialised mechanisms for water uptake and retention in bromeliads that define species' niches (Reyes-García et al., 2012). Conservative stomatal behaviour is a critical component of water retention, and is often associated with CAM in the Bromelioideae. As we hypothesised, the C_3 tank-epiphyte species examined in this investigation showed both high sensitivity of steady-state stomatal conductance to VPD- sufficient to generate a feedforward effect of VPD on transpiration- and rapid stomatal kinetics in response to changes in VPD. This combination may maximise water conservation in dynamic canopy microenvironments, helping plants to maintain a positive water balance. If leaf water potential begins to decline during episodes of low water availability or high evaporative demand, the stomata also close sooner than those of terrestrial species, which may be of particular importance because of their lower internal hydraulic capacitance (Males & Griffiths, 2017a). These C_3 tank-epiphyte

bromeliads therefore exhibit a multifaceted stomatal water-conservation syndrome. Even this may not be sufficient to allow *N. innocentii* to occupy all but the most humid forest microhabitats, since it shows a strong decline in WUE as VPD rises. Indeed, this species is most abundant in hyper-humid riparian forest vegetation (Fischer & Araujo, 1995). Similarly, the steep decline in WUE observed after an initial increase with rising VPD for both *G. monostachia* and *L. glutinosa* could represent a physiological threshold on bioclimatic tolerance. We have observed that *L. glutinosa* is certainly restricted to very wet forest types in its native range, while *G. monostachia* may escape this limitation somewhat by its ability to switch to CAM photosynthesis and stomatal rhythms when challenged by drought or high light. The extent to which these findings hold true for exposure-demanding C_3 tank-epiphytes (e.g. *Vriesea procera* (Mart. ex Schult. & Schult.f.) Wittm.) should be a priority for future research.

While the inferences drawn here are based on measurements made on mature plants, the heteroblastic development of rosette form in many tank bromeliads means that juvenile plants are morphologically and physiologically distinctive (cf. Meisner & Zotz, 2012). Adams & Martin (1986) showed that juvenile and mature plants of *Tillandsia deppeana* Steud. differed in their humidity responses, suggesting that stomatal sensitivity can change within individuals between developmental stages. There is evidence to show that this change in whole-plant physiology is intimately associated with plant size, and occurs through a process of gradual ontogenetic drift rather than abrupt transitions (Schmidt & Zotz, 2001; Meisner et al., 2013). Further work will have to be performed to definitively ascertain the potential for developmental effects and short- or long-term environmental acclimation to determine stomatal responses to humidity in different species. While no data are yet available, it is possible that the blue light sensitivity of stomata of tank-epiphytes is also high, particularly among those that are adapted to forest environments where light availability is concentrated in sun-flecks (Tinoco-Ojanguren & Pearcy, 1992; Campany et al., 2016). However, all other vascular epiphytes studied so far show slow stomatal opening in response to sunflecks (Zotz & Mikona, 2003; Zhang et al., 2009), minimising water expenditure but also limiting their carbon gain during sunfleck exposure. The question of whether the same paradigm holds true for the bromeliads is another area ripe for investigation. Ultimately, it would be desirable to build a comprehensive model of the environmental sensitivity of physiological processes in leaves of bromeliad species of contrasting ecophysiological strategies.

An important avenue for future research will be the characterisation of stomatal sensitivity in CAM bromeliads. This would not only represent a logical extension of the work presented here, but could also shed light on a neglected aspect of the evolution and ecophysiology of CAM plants (Males & Griffiths, 2017b). A comparison of stomatal sensitivity of closely-related C_3 and CAM taxa would be

particularly interesting, perhaps in the morphologically-similar C_3 and CAM xeromorphic terrestrials among the early-diverging Bromelioideae. Alternatively, one could explore trends in stomatal sensitivity associated with the transition from the tank habit and C_3 photosynthesis to the CAM and the remarkable atmospheric habit in the large genus *Tillandsia*. Some of the earliest work on the functional biology of CAM stomata was performed on *Tillandsia* species (Lange & Medina, 1979), and there is now a need to come full circle and revisit these species in greater depth. A rough calculation (see Supporting Information File 1) based on the data of Lange & Medina (1979) suggests that the stomatal sensitivity to VPD in the atmospheric epiphyte *T. recurvata* could be significantly stronger than that measured in the C_3 tank-epiphytes here. Particularly strong stomatal sensitivity would be advantageous to pulse-driven atmospherics making use of transiently available humidity for water-uptake and 'low-cost' gas exchange. Lange & Medina (1979) showed that for *T. recurvata* net assimilation rate closely tracked experimental variation in VPD during the course of the dark period, and it seems likely that strong stomatal sensitivity to humidity and rapid stomatal kinetics underlie this pattern. Lüttge et al. (1986a) have also noted close tracking of ambient humidity by stomatal conductance in the CAM tank-epiphyte *Aechmea aquilega* (Salisb.) Griseb. in Trinidad. Similar effects could be obtained in the light period during Phase IV (late-afternoon) gas exchange by *T. recurvata* (Lange & Medina, 1979), which is consistent with a shared basis for stomatal sensitivity to humidity in the light and in the dark, and therefore probably between C_3 and CAM species. If further light could be shed on the structural basis of quantitative variation in stomatal sensitivity between and among C_3 and CAM species, our understanding of the role of structure-function relationships in the evolutionary history of the bromeliads would be greatly enhanced. Such insights would be timely given their relevance to growing interest in modifying stomatal kinetics to improve WUE in crops (Lawson & Blatt, 2014; McAusland et al, 2016).

The structural basis of variation in stomatal sensitivity

Our data provide tantalising insights into the possible structural basis of differences in stomatal responses to humidity in C_3 bromeliads. There is extensive evidence for the importance of genetically-fixed and plastic stomatal morphological traits in determining gas exchange characteristics in other plant groups (e.g. Franks & Farquhar, 2007; Roth-Nebelsick et al., 2013; Chen et al., 2017). Although the variety of stomatal morphologies in the Bromeliaceae have long been recognised and over five decades ago Tomlinson (1969) postulated ideas regarding their significance since for gas exchange, no concrete advances have thus far been made in this area. This

investigation provided an opportunity to seek evidence for the hypothesis that modifications to various aspects of stomatal morphology could be associated with distinctive functionality.

In the Bromeliaceae, modifications to stomatal complex morphology are always found in association with greater guard cell size and pore length and reduced stomatal density relative to the simple, unmodified arrangement seen in *Pitcairnia* species. This complicates interpretation of measurements of stomatal responses, since stomatal size and density have both been implicated in variation in leaf-level conductance, (Drake et al., 2013; Fanourakis et al., 2014; Franks et al., 2015; Monda et al., 2016). Despite their higher pore lengths, the observed maximal stomatal apertures of the tank-epiphytes were lower than for the *Pitcairnia* species with unmodified stomatal morphology. It is possible that there is a novel mechanism for limiting stomatal aperture in the tank-epiphytes, which could relate either to a modification of guard cell anatomy or an interaction with the underlying hypodermal cells. Alternatively, the energetic and osmotic costs of stomatal opening may be too great for nutrient-limited tank-epiphytes to achieve theoretical maximal conductance.

The observation of higher sensitivity in bromeliad lineages with greater stomatal size and reduced stomatal density bucks the trend among the angiosperms, where studies have generally identified correlations in the opposite direction (Hetherington & Woodward, 2003; Franks & Beerling, 2009; Drake et al., 2013) and Raven (2014) has described a possible energetic and metabolic basis. The decoupling of stomatal pore length and maximal achievable pore width in the bromeliads could partly explain the absence of the usual relationship. Elsewhere, large, low-density stomata that are highly responsive to VPD (but not blue light) are found in the ferns (Brodribb & Holbrook, 2004; McAdam & Brodribb, 2012, 2013; Martins, 2016), but this is likely due to the hydropassive nature of stomatal regulation in ferns. In the bromeliads, we also found a negative relationship between maximal stomatal conductance and stomatal sensitivity rather than the classical positive relationship (Kaufmann, 1982; McNaughton & Jarvis, 1991; Yong et al., 1997; Oren et al., 1999). This unusual trend could be driven by divergent stomatal morphologies with contrasting functional syndromes, and it may be that across the Bromeliaceae as a whole there is a trade-off between investment in stomatal capacity and stomatal sensitivity. Further investigation with wider taxon sampling could explore whether more classical relationships can be recovered within groupings of bromeliad species united by shared stomatal morphology.

Aspects of the spatial distribution of stomata on bromeliad leaves warrant further investigation. For instance, in many tank-epiphytes there is a tendency for pronounced stomatal clustering, which has been associated with reduced gas exchange capacity in *Arabidopsis* (Dow et al., 2014; Lehmann & Or, 2015). As in the monocots more widely (de Boer et al., 2016), amphistomaty (the condition of

having stomata on both adaxial and abaxial surfaces of the leaf) is infrequent in the bromeliads. Only a couple of high-exposure C_3 tank-epiphytes (*Catopsis* spp.) display amphistomatous leaves. The amphistomatous condition has elsewhere been linked with enhanced water use efficiency (Bucher et al., 2017), which could explain why *Catopsis* spp. are able to survive under surprisingly extreme environmental conditions for C_3 plants. The relationship in these species between amphistomaty and stomatal responses to VPD and other factors should be studied further. Additionally, the relationship between leaf age and stomatal sensitivity could be investigated in future, since there is evidence from other rosette plants (e.g. *Arabidopsis*) that stomata in mature leaves are more sensitive to ABA than stomata of younger leaves (Pantin et al., 2013a,b). Given the numerous correlations between leaf age (and leaf region age) and aspects of physiological function in the bromeliads (e.g. Popp et al., 2003; Takahashi et al., 2007), it would be surprising if such a relationship did not occur in these plants too.

Structural modifications of the stomatal complex itself may also be relevant for the strength of the response elicited by a change in VPD. In xeromorphic terrestrial species, stomata are equipped with a pair of large subsidiary cells. By analogy with their counterparts in the grasses, these subsidiary cells may enhance the efficiency of guard cell movements through osmotic coupling and mechanical antagonism (Franks and Farquhar, 2007). It has also previously been mooted that the hypodermal cells underlying the guard cells in these species could play a more direct mechanical role in changes in g_{sw} (Tomlinson, 1969). One possible mechanism would be that if the water potential of the hypodermal cells is in equilibrium with water vapour in the stomatal pore, they could act as an additional hydropassive guard-cell-like resistor. Under high humidity, the hypodermal cells could gain turgor pressure and move away from the lumen of the pore. When humidity falls, the hypodermal cells could lose turgor pressure and fall back into the pore, reducing stomatal conductance. Alternatively, the hypodermal cells could be mechanically engaged with the guard cells such that increasing guard cell turgor causes the hypodermal cells to swing back from the lumen of the pore, and decreasing guard cell turgor draws them in to enhance resistance to gas exchange. Interestingly, although the xeromorphic terrestrial species used here did show strong stomatal sensitivity to humidity, their responses to changes in VPD were relatively slow and involved greater WWR-associated water loss than was observed in either lithophytes or tank-epiphytes. This could be indicative of biochemical or biophysical limitations on the rate of change of stomatal aperture such that the strongest declines in conductance in response to VPD also take the greatest length of time.

The stomatal behaviour of the tank-epiphyte species used here was highly distinctive, combining an apparent feedforward effect of VPD on transpiration driven by strong stomatal sensitivity as well as rapid kinetics. The basis of apparent feedforward responses remain obscure. While models of

stomatal sensitivity to VPD based on hydropassive processes (i.e. equilibration of the water potential of guard cells and/or adjacent tissues with air in the stomatal pore) are often effective at predicting changes in steady-state g_{sw} and provide insights into anatomical determinants of stomatal function (e.g. Peak & Mott, 2011; Mott & Peak, 2013), they do not capture transient WWR effects or the feedforward effect, as discussed by McAdam & Brodribb (2015). Abscisic acid (ABA), a plant growth regulator already known to be an integrating hub in stomatal signalling, has recently been implicated as a critical regulator of the stomatal VPD response in the angiosperms (Bauer et al., 2013; McAdam & Brodribb, 2015; McAdam et al., 2016). Modelling and research in other plant groups has shown that feedforward responses may be associated with changes in leaf hydraulic conductance (Dewar, 2002; Buckley, 2005), or, interestingly, low root hydraulic conductance (Sadok & Sinclair, 2010; Ocheltree et al., 2014). Since the hydraulic conductance of the roots of tank-epiphytic bromeliads, which function primarily as holdfasts, is presumably very low, the same relationship probably holds across the bromeliads. Relative to lithophytes, the tank-epiphytes showed a strong restriction of the duration and intensity of the WWR, but not the RWR, which could imply that different factors are involved in determining the speed of the WWR and the RWR. Further modelling and empirical work will be needed to establish a full understanding of how different structural and biochemical factors interact to determine these traits, building on preliminary evidence that suggests that the initiation of the WWR may be controlled by hydropassive factors and the RWR by hydroactive factors (McAdam et al., 2016).

Taking a broad view of bromeliad evolution, it is striking to note that all of the major transitions in growth form and bioclimatic occupancy appear to have coincided closely with changes in stomatal morphological traits (Males, 2016). The stomatal morphological syndrome of the xeromorphic terrestrial species considered in this study has evolved convergently in multiple lineages with similar ecology (including *Hechtia* and the Xeric Clade Pitcairnioideae; Tomlinson, 1969). A similar syndrome occurs in the CAM atmospheric epiphytes in the genus *Tillandsia*, where stomata might be expected to display similarly conservative responses to environmental stimuli. Meanwhile the tank-epiphyte stomatal morphological syndrome occurs not just in the Bromelioideae and Tillandsioideae (sampled here), but also (in various forms) in understudied lithophytic species of the Navioideae subfamily (Robinson, 1969; Tomlinson, 1969). It is important to note that the species used in this investigation only provide direct insights into the functional stomatal biology of a fraction of the overall diversity of the bromeliad family, and the habitat affinities and environmental tolerances of other important taxa (e.g. non-lithophytic *Pitcairnia* species) may be associated with very different stomatal behaviour. However, on the basis of our observations, we think stomatal innovation could represent an underappreciated, critical player in the evolutionary history of this diverse family. We

suggest that new efforts at phylogenetically-informed analysis of evolutionary trends in mechanistic structure-function interactions in the Bromeliaceae will be repaid by insights of broad relevance in both pure and applied plant physiology.

Conclusions

The highly diverse bromeliad family includes species which display a broad range of growth forms and ecophysiological strategies. There are pronounced differences in stomatal conductance, sensitivity to humidity, and kinetics between C_3 species of contrasting habit and habitat affinity. These differences appear to be of direct relevance to ecological divergences, with mesic tank-epiphyte species showing the lowest stomatal conductance, strongest steady-state responses to humidity, and fastest kinetics. Through stomatal and perhaps also non-stomatal effects, VPD has strongly species-specific effects on intrinsic WUE. The distinctive properties of stomata of different bromeliad groups are strongly linked with stomatal morphological traits, which offer many opportunities for further structure-function characterisation. The possible role of stomatal specialisation in adaptive radiation within specific phylogenetic lineages and functional groups would provide fertile ground for future research.

Acknowledgements

JM received funding from Natural Environment Research Council award 1359020.

References

Adams W.W. & Martin C.E. (1986) Physiological consequences of changes in life form of the Mexican epiphyte *Tillandsia deppeana* (Bromeliaceae). *Oecologia* **70**, 298-304.

Bauer H., Ache P., Lautner S., Fromm J., Hartung W., Al-Rasheid K.A.S., Sonnewald S., Sonnewald U., Kneitz S., Lachmann N. et al. (2013) The stomatal response to reduced relative humidity requires guard-cell autonomous ABA synthesis. *Current Biology* **23**, 53-57.

Benzing D.H. (2000) Bromeliaceae: Profile of an Adaptive Radiation. Cambridge: Cambridge University Press.

de Boer H.J., Price C.A., Wagner-Cremer F., Dekker S.C., Franks P.J. & Veneklaas E.J. (2016) Optimal allocation of leaf epidermal area for gas exchange. *New Phyologist* **210**, 1219-1228.

Brodribb T.J. & Holbrook N.M. (2004) Stomatal protection against hydraulic failure: a comparison of coexisting ferns and angiosperms. *New Phytologist* **162**, 663-670.

Buckley T.N. (2005) The control of stomata by water balance. *New Phytologist* **168,** 275-292.

Buckley T.N. & Mott K.A. (2013) Modelling stomatal conductance in response to environmental factors. *Plant, Cell & Environment* **36,** 1691-1699.

Buckley T.N., Mott K.A. & Farquhar G.D. (2003) A hydromechanical and biochemical model of stomatal conductance. *Plant, Cell & Environment* **26**, 1767-1785.

Buckley T.N., Sack L. & Gilbert M.E. (2011) The role of bundle sheath extensions and life form in stomatal responses to leaf water status. *Plant Physiology* **156**, 962-973.

Bunce J.A. (1997) Does transpiration control stomatal responses to water vapor pressure deficit? *Plant, Cell & Environment* **20**, 131-135.

Butcher D. & Gouda E. (2017) The new Bromeliad Taxon List. Available online: http://botu07.bio.uu.nl/bcg/taxonList.php

Campany C.E., Tjoelker M.G., von Caemmerer S. & Duursma R.A. (2016) Coupled response of stomatal and mesophyll conductance to light enhances photosynthesis of shade leaves under sunflecks. *Plant, Cell & Environment* **39**, 2762-2773.

Chaves C.J.N., Dyonisio J.C. & Rossatto D.R. (2016) Host trait combinations drive abundance and canopy distribution of atmospheric bromeliad assemblages. *AoB Plants* **8**, plw010.

Chen Z.-H., Chen G., Dai F., Wang Y., Hills A., Ruan Y.-L., Zhang G., Franks P.J., Nevo E. & Blatt M.R. (2017) Molecular evolution of grass stomata. *Trends in Plant Science* **22**, 124-139.

Crayn D.M., Winter K., Schulte K. & Smith J.A.C. (2015) Photosynthetic pathways in Bromeliaceae: phylogenetic and ecological significance of CAM and C_3 based on carbon isotope ratios for 1893 species. *Botanical Journal of the Linnean Society* **178**, 169-221.

Dewar R.C. (2002) The Ball-Berry-Leuning and Tardieu-Davies stomatal models: synthesis and extension within a spatially aggregated picture of guard cell function. *Plant, Cell & Environment* **25**, 1383-1398.

Dow G.J., Berry J.A. & Bergmann D.C. (2014) The physiological importance of development mechanisms that enforce proper stomatal spacing in *Arabidopsis thaliana*. *New Phytologist* **201**, 1205-1217.

Drake P.L., Froend R.H. & Franks P.J. (2013) Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* **64**, 495-505.

Fanourakis D., Giday H., Milla, R., Pieruschka R., Kjaer K.H., Bolger M., Vasilevski A., Nunes-Nesi A., Fiorani F. & Ottosen C.-O. (2014) Pore size regulates operating stomatal conductance, while stomatal densities drive the partitioning of conductance between leaf sides. *Annals of Botany* **115**, 555-565.

Farquhar G.D. (1978) Feedforward responses of stomata to humidity. *Australian Journal of Plant Physiology* **5**, 787-800.Fischer E.A. & Araujo A.C. (1995) Spatial organization of a bromeliad community in the Atlantic rainforest, south-eastern Brazil. *Journal of Tropical Ecology* **11**, 559-567.

Franks P.J. & Beerling D.J. (2009) Maximum leaf conductance driven by CO_2 effects on stomatal size and density over geological time. *Proceedings of the National Academy of Sciences of the USA* **106**, 10343-10347.

Franks P.J. & Farquhar G.D. (2007) The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiology* **143**, 78-87.

Franks P.J., Doheny-Adams T.W., Britton-Harper Z.J. & Gray J.E. (2015) Increasing water-use efficiency through genetic manipulation of stomatal density. *New Phytologist* **207**: 188-195.

Freschi L., Takahashi C.A., Cambui C.A., Semprebom T.R., Cruz A.B., Mioto P.T., Versieux L.M., Calvente A. (2010) Specific leaf areas of the tank bromeliad *Guzmania monsotachia* perform distinct functions in response to water shortage. *Journal of Plant Physiology* **167**, 526-533.

Giliberto J., Estay H. (1978) Seasonal water stress in some Chilean matorral shrubs. *Botanical Gazette* **139**, 236-240.

Givnish T.J., Barfuss M.H.J., Van Ee. B., Riina R., Schulte K., Horres R., Gonsiska P.A., Jabaily R.S., Crayn D.M., Smith J.A.C. et al. (2011) Phylogeny, adaptive radiation, and historical biogeography in Bromeliaceae: insights from an eight-locus plastid phylogeny. *American Journal of Botany* **98**, 872-895.

Givnish T.J., Barfuss M.H.J., Van Ee. B., Riina R., Schulte K., Horres R., Gonsiska P.A., Jabaily R.S., Crayn D.M., Smith J.A.C. et al. (2014) Adaptive radiation, correlated and contingent evolution, and net species diversification in Bromeliaceae. *Molecular Phylogenetics and Evolution* **71**, 55-78.

Grassi G. & Magnani F. (2005) Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. *Plant, Cell & Environment* **28**, 834-849.

Griffiths H., Lüttge U., Stimmel K.-H., Crook C.E., Griffiths N.M. & Smith J.A.C. (1986) Comparative ecophysiology of CAM and C_3 bromeliads. III. Environmental influences on CO_2 assimilation and transpiration. *Plant, Cell & Environment* **9**, 385-393.

Hetherington A.M. & Woodward F.I. (2003) The role of stomata in sensing and driving environmental change. *Nature* **424**, 901-908.

Johnsson M., Issaias S., Brogardh T. & Johnsson A. (1976) Rapid, blue-light-induced transpiration response restricted to plants with grass-like stomata. *Physiologia Plantarum* **36**, 229-232.

Kaufmann M.R. (1982) Evaluation of season, temperature, and water stress effects on stomata using a leaf conductance model. *Plant Physiology* **69**, 1023-1026.

Lange O.L., Lösch R., Schulze E.-D. & Kappen L. (1971) Responses of stomata to changes in humidity. *Planta* **100**, 76-86.

Lange, O.L. & Medina, E. (1979) Stomata of the CAM plant *Tillandsia recurvata* respond directly to humidity. *Oecologia* **40**, 357-363.

Lawson T. & Blatt M.R. (2014) Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology* **164**, 1556-1570.

Lehmann P. & Or D. (2015) Effects of stomatal clustering on gas exchange. *New Phytologist* **207**, 015-1025.

Lüttge U., Klauke B., Griffiths H., Smith J.A.C. & Stimmel K.-H. (1986) Comparative ecophysiology of CAM and C_3 bromeliads. V. Gas exchange and leaf structure of the C_3 bromeliad *Pitcairnia integrifolia*. *Plant, Cell & Environment* **9**, 411-419.

Lüttge U., Stimmel K.-H., Smith J.A.C. & Griffiths H. (1986) Comparative ecophysiology of CAM and C_3 bromeliads. II. Field measurements of gas exchange of CAM bromeliads in the humid tropics. *Plant, Cell & Environment* **9**, 377-383.

Males J. (2016) Think tank: water relations of the Bromeliaceae in their evolutionary context. *Botanical Journal of the Linnean Society* **181**, 415-440.

Males J. (2017) Secrets of succulence. *Journal of Experimental Botany* doi: 10.1093/jxb/erx096

Males J., Griffiths H. (2017a) Leaf economic and hydraulic divergences underpin ecological differentiation in the Bromeliaceae. *Plant, Cell & Environment* doi: 10.1111/pce.12954

Males J., Griffiths H. (2017b) Stomatal biology of CAM plants. *Plant Physiology* doi: 10.1104/pp.17.00114

Martins S.C.V., McAdam S.A.M., Deans R.M., DaMatta F.M. & Brodribb T.J. (2016) Stomatal dynamics are limited by leaf hydraulics in ferns and conifers: results from simultaneous measurements of liquid and vapour phase fluxes in leaves. *Plant, Cell & Environment* **39**, 694-705.

McAdam S.A.M. & Brodribb T.J. (2012) Fern and lycophyte guard cells do not respond to endogenous abscisic acid. *Plant Physiology* **24**, 1510-1521.

McAdam S.A.M. & Brodribb T.J. (2013) Ancestral stomatal control results in a canalization of fern and lycophyte adaptation to drought. *New Phytologist* **198**, 429-441.

McAdam S.A.M. & Brodribb T.J. (2015) The evolution of mechanisms driving the stomatal response to vapor pressure deficit. *Plant Physiology* **167**, 833-843.

McAdam S.A.M., Sussmilch F.C. & Brodribb T.J. (2016) Stomatal responses to vapour pressure deficit are regulated by high speed gene expression in angiosperms. *Plant, Cell & Environment* **39**, 485-491.

McAusland L., Vialet-Chabrand S., Davey P., Baker N.R., Brendel O. & Lawson T. (2016) Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologist* **211**, 1209-1220.

McNaughton K.G. & Jarvis P.J. (1991) Effects of spatial scale on stomatal control of transpiration. *Agricultural and Field Meteorology* **54**, 279-302.

Meisner K., Winkler U., Zotz G. (2013) Heteroblasty in bromeliads- anatomical, morphological and physiological changes in ontogeny are not related to the change from atmospheric to tank form. *Functional Plant Biology* **40**, 251-262.

Meisner K., Zotz G. (2012) Heteroblasty in bromeliads- its frequency in a local flora and the timing of the transition from atmospheric to tank form in the field. *International Journal of Plant Sciences* **173**, 780-788.

Monda K., Araki H., Kuhara S., Ishigaki G., Akashi R., Negi J., Kojima M., Sakakiara H., Takahashi S., Hashimoto-Sugimoto M. et al. (2016) Enhanced stomatal conductance by a spontaneous Arabidopsis tetraploid, Me-0, results from increased stomatal size and greater stomatal aperture. *Plant Physiology* **170**, 1435-1444.

Mott K.A. & Franks P.J. (2001) The role of epidermal turgor in stomatal interactions following a local perturbation in humidity. *Plant, Cell & Environment* **24**, 657-662.

Mott K.A. & Peak, D. (2013) Testing a vapour-phase model of stomatal responses to humidity. *Plant, Cell & Environment* **36**, 936-944.

Nonami H., Schulze E.-D. & Ziegler H. (1991) Mechanisms of stomatal movement in response to air humidity, irradiance and xylem water potential. *Planta* **183**, 57-64.

Ocheltree T.W., Nippert J.P. & Prasad P.V.V. (2014) Stomatal responses to changes in vapor pressure deficit reflect tissue-specific differences in hydraulic conductance. *Plant, Cell & Environment* **37**, 132-139.

Oren R., Sperry J.S., Katul G.G., Pataki D.E., Ewers B.E., Phillips N., Schäfer K.V.R. (1999) Survey and synthesis of intra- and interspecific variation in stomatal sensitivity to vapour pressure deficit. *Plant, Cell & Environment* **22**, 1515-1526.

Palma-Silva C., Leal B.S.S., Chaves C.J.N. & Fay M.F. (2016) Advances in and perspectives on evolution in Bromeliaceae. *Botanical Journal of the Linnean Society* **181**, 305-322.

Pantin F., Monnet F., Jannaud D., Costa J.M., Renaud J., Muller B., Simonneau T., Genty B. (2013a) The dual effect of abscisic acid on stomata. *New Phytologist* **197**, 65-72.

Pantin F., Renaud J., Barbier F., Vavasseur A., Le Thiec D., Rose C., Bariac T., Casson S., McLachlan D.H., Hetherington A.M. (2013b) Developmental priming of stomatal sensitivity to abscisic acid by leaf microclimate. *Current Biology* **23**, 1805-1811.

Peak, D. & Mott K.A. (2011) A new, vapour-phase mechanism for stomatal responses to humidity and temperature. *Plant, Cell & Environment* **34**, 162-178.

Pittendrigh C.S. (1948) The bromeliad-Anopheles-malaria complex in Trinidad. I- The bromeliad flora. *Evolution* **2**, 58-89.

Popp M., Janett H.-P., Lüttge U., Medina E. (2003) Metabolite gradients and carbohydrate translocation in rosette leaves of CAM and C3 bromeliads. *New Phytologist* **157**, 649-656.

Raven J.A. (2014) Speedy small stomata? *Journal of Experimental Botany* **65**, 1415-1424.

Reyes-García C., Mejia-Chang M. & Griffiths H. (2012) High but not dry: diverse epiphytic bromeliad adaptations to exposure within a seasonally dry tropical forest community. *New Phytologist* **193**, 745-754.

Robinson H. (1969) A monograph on foliar anatomy of the genera *Connellia*, *Cottendorfia*, and *Navia* (Bromeliaceae). *Smithsonian Contributions to Botany* **2**

Roth-Nebelsick A., Fernández V., Peguero-Pina J.J., Sancho-Knapik D., Gil-Pelegrín E. (2013) Stomatal encryption by epicuticular waxes as a plastic trait modifying gas exchange in a Mediterranean evergreen species (*Quercus coccifera* L.). *Plant, Cell & Environment* **36**, 579-589.

Sadok W. & Sinclair T. (2010) Genetic variability of transpiration response of Soybean (*Glycine max* (L.) Mem) shoots to leaf hydraulic conductance inhibitor $AgNO_3$. *Crop Science* **50**, 1423-1430.

Schmidt G., Zotz G. (2001) Ecophysiological consequences of differences in plant size- in situ carbon gain and water relations of the epiphytic bromeliad *Vriesea sanguinolenta*. *Plant, Cell & Environment* **24**, 101-112.

Schulte K., Barfuss M.H.J. & Zizka G. (2009) Phylogeny of Bromelioideae (Bromeliaceae) inferred from nuclear and plastid DNA loci reveals the evolution of the tank habit within the subfamily. *Molecular Phylogenetics and Evolution* **51**, 327-339.

Silvestro D., Zizka G. & Schulte K. (2014) Disentangling the effects of key innovations on the diversification of Bromelioideae (Bromeliaceae). *Evolution* **68**, 63-175.

Takahashi C.A., Ceccantini G.C.T., Mercier H. (2007) Differential capacity of nitrogen assimilation between apical and basal leaf portions of a tank epiphytic bromeliad. *Brazilian Journal of Plant Physiology* **19**, 119-126.

Tinoco-Ojanguren C. & Pearcy R.W. (1992) Dynamic stomatal behavior and its role in carbon gain during lightflecks of a gap phase and an understory *Piper* species acclimated to high and low light. *Oecologia* **92**, 222-228.

Tomlinson P.B. (1969) Anatomy of the Monocotyledons III. Commelinales-Zingiberales. Oxford: Clarendon Press.

Valdebenito H., Stuessy T.F., Crawford D.J. & Silva M.O. (1992) Evolution of *Erigeron* (Compositae) in the Juan Fernandez Islands, Chile. *Systematic Botany* **17**, 470-480.

Vialet-Chabrand S., Matthews J.S.A., Brendel O., Blatt M.R., Wang Y., Hills A., Griffiths H., Rogers S.T. & Lawson T. (2016) Modelling water use efficiency in a dynamic environment: an example using *Arabidopsis thaliana*. *Plant Science* **251**, 65-74.

Wolf A., Anderegg W.R.L. & Pascala S.W. (2016) Optimal stomatal behavior with competition for water and risk of hydraulic impairment. *Proceedings of the National Academy of Sciences of the USA* **113**, E7222-E7230.

Yong J.W.H., Wong S.C. & Farquhar G.D. (1997) Stomatal responses to changes in vapour pressure difference between leaf and air. *Plant, Cell & Environment* **20**, 1213-1216.

Zhang Q., Chen J.-W., Li B.-G., Cao K.-F. (2009) Epiphytes and hemiepiphytes have slower photosynthetic response to lightflecks than terrestrial plants: evidence from ferns and figs. *Journal of Tropical Ecology* **25**, 465-472.

Zizka G., Schmidt M., Schulte K., Novoa P., Pinto R. & König K. (2009) Chilean Bromeliaceae: diversity, distribution, and evaluation of conservation status. *Biodiversity and Conservation* **18**, 2449-2471.

Zotz G., Mikona C. (2003) Photosynthetic induction and leaf carbon gain in the tropical understory epiphyte *Aspasia principisa*. *Annals of Botany* **91**, 353-359.

Tables

Species	Subfamily	Functional group	Habitat
△ *Pitcairnia integrifolia* [L]	Pitcairnioideae	Lithophyte (L)	Tropical moist forest
▲ *Pitcairnia xanthocalyx* [L]	Pitcairnioideae	Lithophyte (L)	Tropical dry forest
● *Puya berteroniana* [X]	Puyoideae	Xeromorphic terrestrial (X)	Subtropical (alpine) scrub
○ *Puya mirabilis* [X]	Puyoideae	Xeromorphic terrestrial (X)	Subtropical (alpine) scrub
■ *Ochagavia elegans* [X]	Bromelioideae	Xeromorphic terrestrial (X)	Subtropical coastal scrub
★ *Guzmania monostachia* [T]	Tillandsioideae	Tank-epiphyte (T)	Tropical/ subtropical moist/dry forest
☆ *Lutheria glutinosa* [T]	Tillandsioideae	Tank-epiphyte (T)	Tropical montane forest
□ *Nidularium innocentii* [T]	Bromelioideae	Tank-epiphyte (T)	Tropical moist forest

Table 1. Summary of ecological properties of species used in this investigation, showing symbols used to identify species in figures.

Species	Stomatal pore length (μm)	± SE	Stomatal density (mm^{-2})	± SE	α_{max} (μ)	± SE	S_α
△ *Pitcairnia integrifolia* [L]	11	0.05	413	12.31	3.80	0.02	0.18
▲ *Pitcairnia xanthocalyx* [L]	10	0.07	438	15.62	3.44	0.04	0.27
● *Puya berteroniana* [X]	54	0.11	70	4.81	5.39	0.01	0.29
○ *Puya mirabilis* [X]	48	0.06	92	3.77	4.70	0.05	0.26
■ *Ochagavia elegans* [X]	49	0.10	16	0.89	4.94	0.03	0.36
★ *Guzmania monostachia* [T]	29	0.08	37	2.26	2.88	0.03	0.61
☆ *Lutheria glutinosa* [T]	34	0.04	16	1.01	2.56	0.04	0.64
□ *Nidularium innocentii* [T]	39	0.12	7	0.22	2.66	0.02	0.61

Table 2. Mean stomatal size, stomatal density, maximal stomatal aperture (α_{max}), and instantaneous sensitivity of stomatal aperture to VPD (S_α) for eight C_3 bromeliad species $n \geq 3$ individuals per species for all measurements. [L] = lithophyte; [X] = xeromorphic terrestrial; [T] = tank-epiphyte.

Species	g_{swmax} (mol m^{-2} s^{-1})	± SE	S_S	A_{max} (μmol m^{-2} s^{-1})	± SE	S_A
△ *Pitcairnia integrifolia* [L]	0.168	0.009	0.158	5.67	0.14	0.084
▲ *Pitcairnia xanthocalyx* [L]	0.139	0.013	0.150	6.61	0.16	0.131
● *Puya berteroniana* [X]	0.262	0.022	0.157	4.22	0.14	0.033
○ *Puya mirabilis* [X]	0.228	0.018	0.157	7.54	0.30	0.133
■ *Ochagavia elegans* [X]	0.250	0.024	0.146	3.62	0.20	0.236
★ *Guzmania monostachia* [T]	0.136	0.005	0.186	5.62	0.19	0.155
☆ *Lutheria glutinosa* [T]	0.110	0.010	0.205	3.40	0.22	0.193
□ *Nidularium innocentii* [T]	0.039	0.002	0.215	3.10	0.17	0.239

Table 3. Mean maximal stomatal conductance to water vapour (g_{swmax}), instantaneous stomatal sensitivity to VPD (S_s), maximal net CO_2 assimilation rate (A_{max}), and instantaneous sensitivity of assimilation rate to VPD (S_A) for eight C_3 bromeliad species. $n \geq 3$ individuals per species for all measurements. [L] = lithophyte; [S] = xeromorphic terrestrial; [T] = tank-epiphyte.

Species	t_{WWR} (s)	± SE	AUC_{WWR} (mol m^{-2})	± SE	t_{RWR} (s)	± SE
△ *Pitcairnia integrifolia* [L]	1210	113.18	100	7.90	1310	93.71
▲ *Pitcairnia xanthocalyx* [L]	1490	139.84	72	5.29	1010	125.85
● *Puya berteroniana* [X]	2560	277.65	219	18.67	2360	199.73
○ *Puya mirabilis* [X]	1950	189.96	138	12.54	2560	310.12
■ *Ochagavia elegans* [X]	1920	103.00	61	7.22	1950	205.04
★ *Guzmania monostachia* [T]	900	37.15	28	3.08	1120	89.55
☆ *Lutheria glutinosa* [T]	910	28.54	12	1.15	1520	137.21
□ *Nidularium innocentii* [T]	670	33.53	3	0.08	1425	166.81

Table 4. Mean species values of parameters describing wrong- and right-way responses of stomatal conductance to water vapour (g_{sw}) to an increase of VPD from 1.3 kPa to 1.8 kPa. t_{WWR} = duration of wrong-way response; AUC_{WWR} = area under stomatal conductance curve during wrong-way response; t_{RWR} = time for completion of right-way response. $n \geq 3$ individuals per species. [L] = lithophyte; [S] = xeromorphic terrestrial; [T] = tank-epiphyte.

Figure 1. Bromeliad species used in this investigation: a) *Pitcairnia integrifolia*; b) *Pitcairnia xanthocalyx*; c) *Puya berteroniana*; d) *Puya mirabilis*; e) *Ochagavia elegans*; f) *Guzmania lingulata*; g) *Lutheria glutinosa*; h) *Nidularium innocentii*. Photos: a,f,g) JM; b) Tony Rodd (CC BY-NC-SA 2.0); c) Sean O'Hara (CC BY 2.0); d,h) Timm Stolten (CC BY-SA 3.0); e) Jardín Botánico Nacional de Chile (CC BY-NC 2.0).

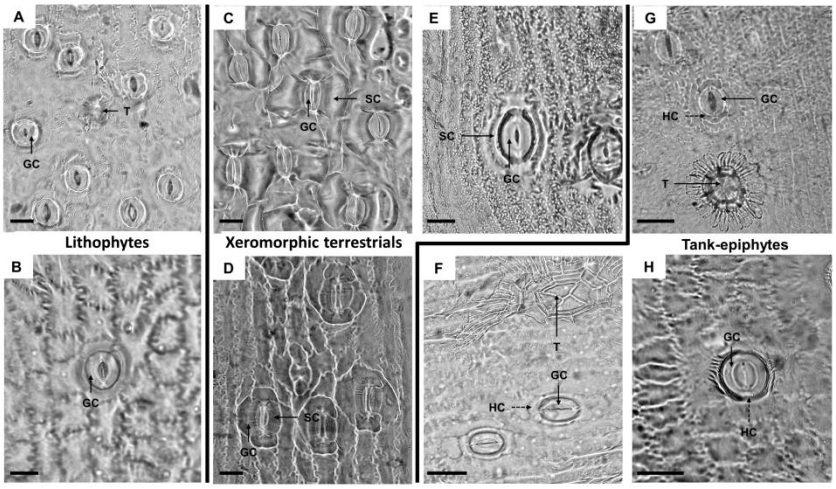

Figure 2 [UPDATED- "ss revised fig 2.png"]. Epidermal impressions showing stomatal complexes for species used in this investigation: a) *Pitcairnia integrifolia*; b) *Pitcairnia xanthocalyx*; c) *Puya berteroniana*; d) *Puya mirabilis*; e) *Ochagavia elegans*; f) *Guzmania lingulata*; g) *Lutheria glutinosa*; h) *Nidularium innocentii*. GC = guard cell; SC = subsidiary cell; HC = hypodermal cell; T = trichome. Scale bars = 10 µm (a-b), 50 µm (c-h).

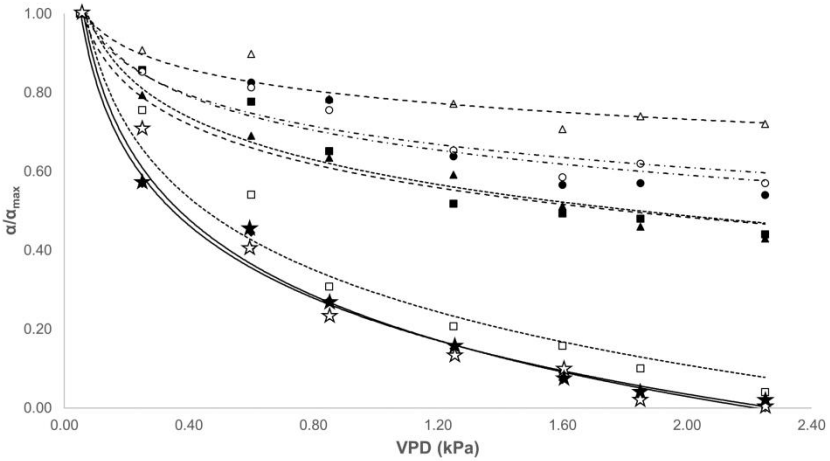

Figure 3. Responses of mean directly-measured stomatal aperture (α) normalised by maximal stomatal aperture (α_{max}) to vapour pressure deficit (VPD). Species key: △ *Pitcairnia integrifolia*; ▲ *Pitcairnia xanthocalyx*; ● *Puya berteroniana*; ○ *Puya mirabilis*; ■ *Ochagavia elegans*; ★ *Guzmania monostachia*; ☆ *Lutheria glutinosa*; □ *Nidularium innocentii*.

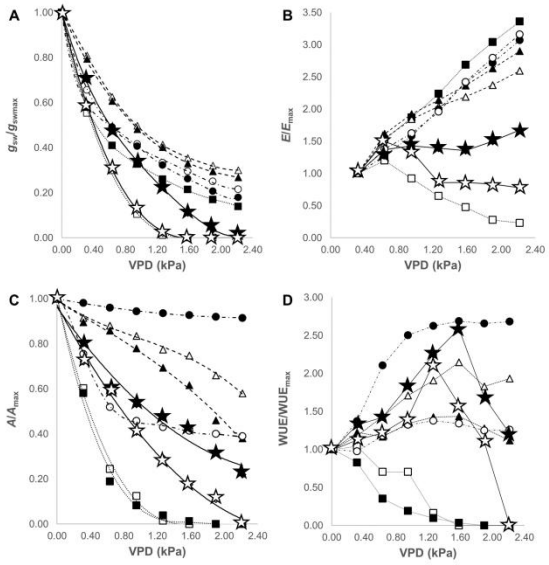

Figure 4. Responses of mean physiological parameters to vapour pressure deficit (VPD). a) Stomatal conductance to water vapour (g_{sw}) normalised by maximum value (g_{swmax}); b) transpiration rate (E) normalised by maximum value (E_{max}); c) net assimilation rate (A) normalised by maximum value (A_{max}); d) water-use efficiency (WUE) normalised by maximum value (WUE_{max}). Species key: △ *Pitcairnia integrifolia*; ▲ *Pitcairnia xanthocalyx*; ● *Puya berteroniana*; ○ *Puya mirabilis*; ■ *Ochagavia elegans*; ★ *Guzmania monostachia*; ☆ *Lutheria glutinosa*; □ *Nidularium innocentii*.

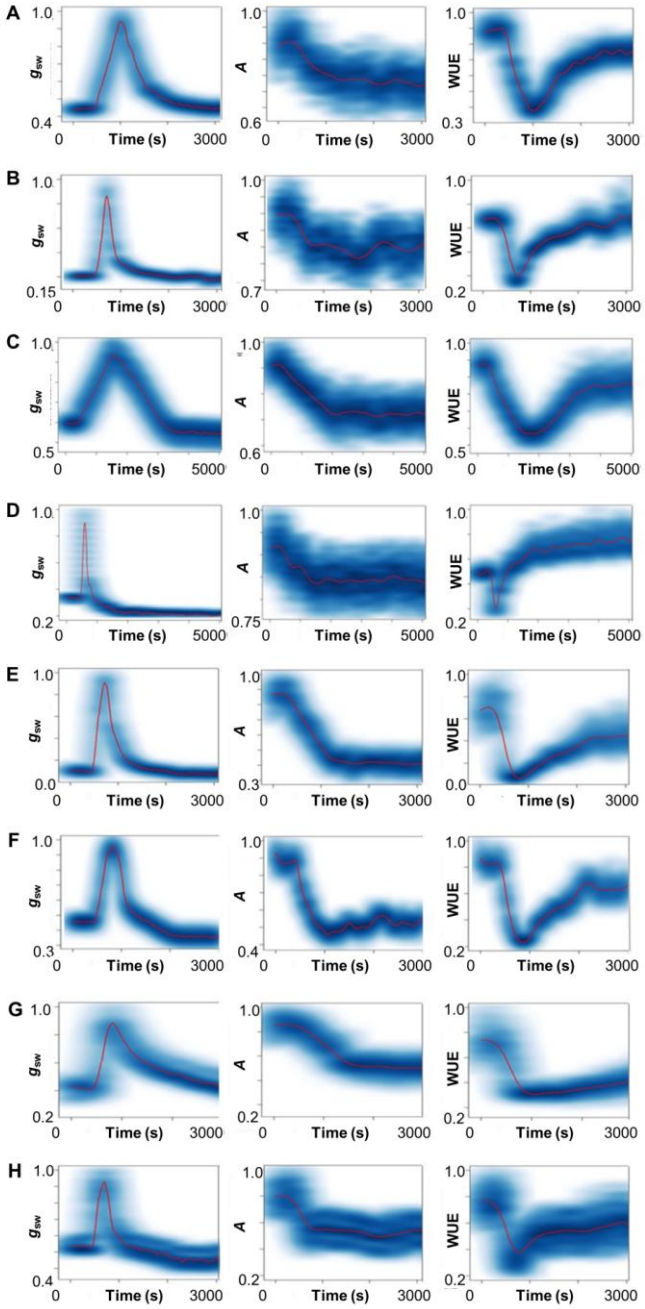

Figure 5. Kinetics of normalised stomatal conductance to water vapour (g_{sw}), net assimilation rate (A), and water-use efficiency (WUE) in response to a step-increase in vapour pressure deficit from 1.3 kPa to 1.8 kPa: a) *Pitcairnia integrifolia*; b) *Pitcairnia xanthocalyx*; c) *Puya berteroniana*; d) *Puya mirabilis*; e) *Ochagavia elegans*; f) *Guzmania lingulata*; g) *Lutheria glutinosa*; h) *Nidularium innocentii*. Blue point clouds represent data from eight repeats per species; red lines are smoothed splines.